Towards optimal identification of carriers of germline mutations involved in mismatch repair

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SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Hereditary nonpolyposis colorectal cancer (HNPCC) is one of the most commonly occurring hereditary conditions that lead to an increased risk for several tumor types, with the highest lifetime risks for colorectal and endometrial cancer. In **Chapter 1** a short historical introduction is given concerning HNPCC. The formulation of clinical criteria in 1991, known as the Amsterdam Criteria, were primarily meant to help research in this field by providing a uniform definition of the disorder, but were soon also used to define families to be HNPCC-families for clinical purposes.[1] These criteria include presence of (1) at least three relatives with histologically verified cancer of the colorectum, one of whom is a first-degree relative of the other two; (2) at least two successive generations affected; (3) at least one case diagnosed under the age of 50 years; and (4) exclusion of familial adenomatous polyposis. The latter is important as HNPCC lacks pathognomonic phenotypic features. Therefore, the diagnosis cannot be made in an individual purely based on individual characteristics.

Soon after the Amsterdam Criteria were formulated, the mismatch repair (MMR) genes *MSH2* and *MLH1* were identified and the association between germline mutations in these genes and HNPCC, as clinically defined, was established.[2-4] More than half of the families fulfilling the Amsterdam Criteria proved to harbor a germline mutation in one of these two genes and what was first a syndrome just by clinical definition, could now be linked to a genetic defect. However, it became clear that only part of the patients and their families that carry a pathogenic *MLH1* or *MSH2* mutation fulfill the Amsterdam Criteria. This is due to a number of factors: the occurrence of extracolonic cancers in mutation carriers, the difficulty to verify the cancer diagnosis in family members that passed away a long time ago, a decreasing average family size and the occurrence, though rare, of *de novo* mutations. On the other hand, large pedigrees may sometimes fulfill the Amsterdam Criteria as the result of accidental clustering of three first-degree relatives with cancer. Germline mutations in another human MMR gene, the *MSH6* gene, were reported for the first time in 1997 in members of two families that did not fulfill the Amsterdam Criteria.[5,6] This was another reason to realize that the Amsterdam Criteria were not sensitive enough to identify all mutation carriers.

Defective MMR function is characterized by microsatellite instability (MSI) and by loss of MMR protein expression in the tumor tissue. Microsatellites are short repetitive base sequences. Tumor cells deficient in post-replication MMR function show an increase in the rate of spontaneous mutations, most readily seen in microsatellites, both in non-coding and in coding sequences, including sequences in genes which are involved in the development and progression of tumors, like the *TGFβRII* and *Bax* genes.[7] Since almost all colorectal cancers from patients with an *MLH1* or an *MSH2* mutation show microsatellite instability[8], MSI analysis has been put forward as a selection criterion for mutation analysis. At an international conference in Bethesda, MD, USA, in 1996 consensus was reached about criteria to identify patients whose tumors should be tested for MSI and the Bethesda criteria were introduced.[9] To ensure uniformity, a reference panel of the most sensitive microsatellite markers was developed and validated to perform MSI analysis in colorectal cancer.[8] Though a valuable tool[10,11], only a small minority of colorectal cancers and other tumors exhibiting microsatellite instability are due to a germline mutation in the *MLH1* or *MSH2* genes.[12] Sporadic cases of microsatellite instable cancers may be due to somatic inactivation of MMR genes.

Besides MSI, immunohistochemical analysis for the two MMR proteins MLH1 and MSH2 has been widely introduced[13-15], and the application of the MSH6 antibody to

determine MSH6 protein expression is increasing.[16-19] The two methods, MSI analysis and immunohistochemical analysis, are highly sensitive and provide the opportunity to sort out which patient should be further analyzed for germline MMR gene mutations.[15] Advantages of immunohistochemistry over MSI analysis include its more widespread availability, lower costs and the direction it gives for mutation analysis.

Identifying germline MMR gene mutations is important as mutation carriers and their families are eligible for schemes for periodic screening to detect neoplasms at the earliest possible stages. It has been shown that periodic colonoscopy is useful and drastically reduces colorectal cancer mortality.[20,21] If one aims to detect as many carriers with germline MMR gene mutations as possible, the Amsterdam Criteria appeared to be not sensitive enough as mentioned above, and other ways of identification of patients and families were needed. Therefore, other sets of clinical criteria besides the Amsterdam Criteria were formulated in the past years to detect patients with a relevant germline mutation with a high sensitivity, and acceptable specificity and positive and negative predictive values. These criteria are reviewed and discussed in **Chapter 2.** Each of the proposed set of clinical criteria was less stringent than the Amsterdam Criteria, but the yield of mutations was usually lower than when using the Amsterdam Criteria. Furthermore, in most studies the criteria were evaluated in only a small number of subjects. It is apparently very difficult to formulate optimal criteria to select those patients that are most suspect for having a germline MMR gene mutation. The original Amsterdam Criteria were extended in 1999 to include those extracolonic cancers that showed the highest relative risk to occur in mutation carriers, i.e. endometrial cancer, small bowel cancer and transitional cell cancer of the renal pelvis and ureter. These criteria were called the Amsterdam Criteria II or revised Amsterdam criteria.[22] According to a recent study the relative mutation detection rate of these criteria was about the same compared to the old criteria (50% (109/217) versus 52% (101/193) respectively).[23]

The goal of this thesis was threefold as described in **Chapter 1**. First of all, the prevalence of germline mutations in the *MLH1, MSH2* and *MSH6* genes was determined in different patient populations, suspected of HNPCC. Secondly, the outcome of the MSI analysis and immunohistochemical analysis of the three MMR proteins were studied in relation to each other, to the results of the mutation analysis and to the clinical data and family history data in order to determine independent high risk variables for detecting putative MMR gene mutation carriers. Finally, the results of this study should help to formulate accurate and simple criteria for use in the clinical practice. Patients could participate in the study when fulfilling one of the following criteria: (1) patients with colorectal cancer or endometrial cancer diagnosed under 50 years of age; (2) patients with an HNPCC-related cancer and a first-degree relative with colorectal cancer or endometrial cancer, or vice versa, one of them diagnosed under 50 years of age; (3) patients with two or more HNPCC-related cancers, irrespective of age or; (4) patients with a colorectal adenoma or atypical endometrial hyperplasia that have a first-degree relative with colorectal cancer or endometrial cancer, both diagnosed at an age younger than 50 years. HNPCC-related cancers as defined in this study were colorectal cancer, endometrial cancer, cancer of the small bowel, the stomach, the pancreas, the biliary tract and the ovaries and transitional cell cancer of the renal pelvis, ureter and bladder. Three large groups of patients emerged during the course of the study, namely patients with colorectal cancer and patients with endometrial cancer, all diagnosed under the

age of 50 year (criterion 1), and patients with multiple HNPCC-related cancers, irrespective of age (criterion 3). Further analyses were focused on these three groups.

For two reasons, only the medical history concerning cancer of first-degree relatives was included to make associations and calculations. Firstly, in clinical practice, selection criteria should be as simple as possible. Secondly, medical histories of first-degree relatives can be verified better than those of, for example, grandparents. However, to identify Amsterdam families among the families of the patients in our study, data on family history were completed up to the third degree, but there may have been some bias because of incorrect information. Recently, the reliability of family history was investigated in a total of 1200 patients with colorectal cancer.[24] That study suggested that family studies on HNPCC are not reliable unless the diagnoses of family members are verified from official sources. Both false-positive family and false-negative family histories for the Amsterdam Criteria II were detected. Thus, verification of medical histories appears of vital importance.

In **Chapter 3**, the patients with colorectal cancer, diagnosed under the age of 50 years are reported. Young age at diagnosis of colorectal cancer is an important, independent predictor of a mutation in the *MLH1* or *MSH2* gene[25] and is included in the Amsterdam Criteria II.[22] To identify better criteria to select young colorectal cancer patients for mutation analysis in future, predictive values of first-degree family history, tumor MSI analysis and immunohistochemical analysis of the three MMR proteins MLH1, MSH2 and MSH6 in predicting pathogenic germline mutations were calculated and multivariate analysis was performed. In 14 out of 224 young colorectal cancer patients (6%) a mutation was found leading to truncation of the protein product and, therefore, considered as pathogenic. The sensitivity of the Amsterdam Criteria II appeared to be low (29%) as only 4 of the 14 mutations detected were found amongst the 14 patients from (assumed) Amsterdam families. First-degree family history as well as MSI analysis and immunohistochemical analysis were good predictors to select patients possibly having germline mutations. When selection would be based on family history alone, however, three mutation carriers would have been missed, whereas both MSI and immunohistochemical analysis would have missed one carrier.

Immunohistochemical analysis had a seemingly higher positive predictive value than MSI-analysis, implying that less mutation analyses would be needed when relying upon the results of the immunohistochemical analysis. Multivariate analysis for the aforementioned variables revealed that immunohistochemical analysis is the single best predictor to select patients for the analysis of mutations as defined above. Thus, in patients diagnosed with colorectal cancer before the age of 50 years, MMR protein staining should be performed, in particular when there is a positive first-degree family history for HNPCC-related cancer(s). When staining of at least one MMR protein is absent, patients should then be offered mutation analysis of the non-expressed gene(s). The relatively easy immunohistochemical technique should become generally available to identify the large majority of MMR gene mutation carriers among young colorectal cancer patients.

The same procedure that was applied for young colorectal cancer patients was also used for patients with multiple HNPCC-related cancers. **Chapter 4** describes the results of germline MMR gene mutation analysis in 96 such patients in relation to age at diagnosis, tumor types, family history, and tumor MSI and MMR protein expression in the tumors. Fifteen pathogenic germline mutations leading to a truncated protein product were detected (15/96, 16%). All mutation carriers had their first tumor diagnosed under the age of 60 years; 10 of them were younger than 50 years at diagnosis. All had at least one colorectal cancer. Four out of six *MSH6* mutations were detected in patients with their first tumor diagnosed

above age 50 years. Twelve of the mutation carriers had one or more first-degree relatives with an HNPCC-related cancer, predominantly colorectal or endometrial cancer. In six of 12 patients from families fulfilling the Amsterdam Criteria II a germline mutation was present. MSI analysis missed three *MSH6* mutation carriers; immunohistochemical analysis did not miss any mutation. Based on our study results, it is concluded that in multiple HNPCC-related cancer patients, selection for mutation analysis is best based on age at diagnosis of the first tumor under 60 years of age, the presence of at least one colorectal cancer and immunohistochemical analysis of the tumors for the MMR proteins.

In **Chapter 5** the question was asked if adrenocortical adenocarcinoma should be added to the HNPCC tumor spectrum. The question arose when one of the multiple cancer patients described in Chapter 4 with an ovarian adenocarcinoma, three metachronous colorectal cancers and an adrenocortical adenocarcinoma turned out to be an *MSH2* truncating mutation carrier. Since the adrenal tumor was microsatellite-stable and no general loss of MSH2 protein in the adrenal tumor could be demonstrated, the development of this cancer should be considered not to be associated with the germline *MSH2* mutation. Based on the findings in this patient, there is no reason to include adrenocortical adenocarcinoma in the HNPCC-tumor spectrum.

In **Chapter 6** published studies on possible hereditary factors in the development of endometrial cancer, especially in patients diagnosed under the age of 50 years, are reviewed. In some patients with endometrial cancer, other cancers, such as colorectal cancer or ovarian cancer, occur synchronously or metachronously. This is, besides a positive family history and a young age at diagnosis, indicative for a hereditary predisposition. HNPCC is now known to be responsible for a small subset of endometrial cancers. This cancer is the second most commonly occurring cancer in HNPCC. The question was which subset of young endometrial cancer patients should be referred for genetic counseling. The answer is given in **Chapter 7** and **Chapter 8**. In **Chapter 7**, use of immunohistochemical analysis of MLH1 and MSH2 proteins in selecting endometrial cancer patients for mutation analysis was examined. For this purpose, immunohistochemical analysis was performed on normal and (pre)malignant endometrial samples from three groups of patients: 1. endometrial cancer patients with a proven pathogenic *MLH1* or *MSH2* germline mutation, 2. endometrial cancer patients without such mutation but with a family, fulfilling the Amsterdam Criteria II, and 3. endometrial cancer patients, not fulfilling these HNPCC criteria, diagnosed under the age of 50 years. A strong relation could be shown in our study between presence of *MLH1* and *MSH2* germline mutations leading to a truncating protein product and the loss of corresponding protein expression in endometrial cancer from MMR gene mutation carriers as found by immunohistochemical analysis. This justifies the use of this type of analysis of these tumors as a pre-screening method for mutation detection in clinically defined HNPCC families. The loss of MLH1 or MSH2 protein was also common in endometrial hyperplasia of MMR gene mutation carriers as well as in endometrial hyperplasia of young endometrial cancer patients in which no mutation had been detected; this indicates that loss of function of the MMR proteins occurs early in carcinogenesis. In **Chapter 8** we describe mutation analysis in an extended series of 58 endometrial cancer patients diagnosed under the age of 50 years who were unselected for family history. In five of them a truncating mutation was detected. In addition to MLH1 and MSH2 staining, performed in the study described in chapter 7, also MSH6 immunohistochemical analysis was performed. In patients from families fulfilling the revised Amsterdam Criteria the mutation rate was 50%. All five mutation carriers had a strongly positive family history (four of the five patients came from families fulfilling the

revised Amsterdam Criteria). Immunohistochemical analysis predicted all truncating mutations. MSI analysis missed one *MSH6* mutation carrier. As a pre-screening method for mutation analysis in young endometrial cancer patients immunohistochemical analysis in such patients with at least one first-degree relative with an HNPCC-related cancer is valuable while MSI analysis in this series of young endometrial cancer patients has not proved to have additional value over immunohistochemical analysis.

The *MSH6* gene is one of the MMR genes involved in HNPCC. In families fulfilling the Amsterdam Criteria the proportion of *MSH6* germline mutations has been reported to be lower than the proportion of *MLH1* and *MSH2* mutations.[26,27] An explanation for the lower frequency of *MSH6* mutations in such families may be the fact that because of compensation by other MMR proteins loss of the MSH6 function only causes a partial loss of MMR functioning and that for (a proportion of) *MSH6* mutations the penetrance of the genetic predisposition may be lower than in case of mutations of *MLH1* or *MSH2*. In order to further define the molecular and clinical implications of *MSH6* germline variants, all patients with truncating and missense germline *MSH6* mutations that we detected are described in **Chapter 9.** Three hundred and sixteen individuals with (suspicion of) HNPCC were analyzed for *MSH6* germline mutations. Five different truncating *MSH6* mutations, of which one was detected seven times, were found in 12 index patients and ten *MSH6* variants with unknown pathogenicity were found in another 13 index patients. For the 25 index patients and eight of their relatives with *MSH6* variants, molecular and clinical features are described. In classical Amsterdam families the prevalence of *MSH6* mutations was about 10%; most families of *MSH6* mutation carriers, however, were atypical and belonged to what could be called the category of suspected HNPCC. In non-Amsterdam families, the prevalence of *MSH6* variants was estimated to be about the same as the prevalence of *MLH1* or *MSH2* variants. A substantial proportion of tumors in *MSH6* mutation carriers did not show a high degree of MSI. Twelve out of the total of 18 tumors from the twelve pathogenic *MSH6* mutation carriers showed absence of the MSH6 protein by immunohistochemical analysis. Female carriers of *MSH6* mutations appeared to be at a high risk for endometrial cancer. The majority of the colorectal cancers were localized distally in the colon. Missense variants in *MSH6* were about as common as truncating mutations and, as there were no differences in molecular and clinical characteristics, it is suggested that a considerable number of these missense variants are also pathogenic. We conclude that for all HNPCC-suspected patients *MSH6* mutation analysis should be considered and that neither MSI nor immunohistochemical analysis should be a definite selection criterion for *MSH6* mutation analysis.

Besides the *MLH1, MSH2* and *MSH6* genes two additional genes that have recently been identified were analyzed for their possible role in HNPCC. One of these is *EXO1*. **Chapter 10** describes the detection of one splice-site mutation in an Amsterdam family and eight missense *EXO1* gene variants in 13 patients suspected of HNPCC. Analysis of DNA from tumors from the variant carriers revealed only one case without *EXO1* allelic loss, whereas 12 tumors had lost the allele with the variant sequence and had retained the normal allele. As this seems in contradiction with current ideas of cancer development by MMR gene defects several models may be possible to explain the afore-mentioned, among them a haploinsufficiency model. A haploinsufficiency effect of *EXO1* in the tissues could cause cells that only retain a mutant allele to be non-viable. Cells that only retain a wild-type allele

would survive, but in some tissues the amount of product from this single allele may be insufficient to prevent cancer development.

In **Chapter 11** it was shown that the gene *MLH3* might play a role in patients, suspected of HNPCC. Ten different germline *MLH3* variants, one frameshift mutation and nine missense variants, were detected in 12 patients with HNPCC suspicion. After performing MSI analysis with mono-, di- tri- and tetranucleotide markers, mononucleotide markers did not appear to be very informative. Using the five most informative repeat markers, including only one dinucleotide marker from the consensus panel of markers, the majority of the tumors showed a high degree of MSI. With immunohistochemical analysis somatic inactivation of the *MSH2, MLH1* and *MSH6* gene was ruled out.
According to our present results, the *EXO1* and *MLH3* genes do not seem to play a significant role in HNPCC. Predominantly missense variants were found in these genes. Functional assays will be needed to clarify the role of these probably low-penetrant mutations in typical and atypical HNPCC families.

GENERAL DISCUSSION

In patients with colorectal cancer and endometrial cancer diagnosed under the age of 50 years, the prevalence of pathogenic germline mutations was less than 10% while this was 16% in patients with multiple HNPCC-associated cancers. In the three aforementioned groups a positive first-degree family history increased the risk of having a germline mutation markedly. The Amsterdam Criteria II showed to have a low to moderate sensitivity, at least in the groups of multiple cancer patients and young colorectal cancer patients. This was different for young endometrial cancer patients as four of the five mutations were found in patients from Amsterdam II families. This is plausible, as the penetrance of endometrial cancer in HNPCC families is lower than that of colorectal cancer, meaning that the presence of endometrial cancer in HNPCC-suspected families strongly indicates the presence of a germline MMR gene mutation, as was already shown in the study of Wijnen *et al* (1998).[25]
When the results of the three patients groups that we studied are combined (n=344 patients), in about one third (8/25) of all families fulfilling the Amsterdam criteria II (AC II) pathogenic mutations occurred. The remaining 16 mutations were found in 319 non-Amsterdam families. Thus, the sensitivity of the Amsterdam Criteria II is 33.3% (8/24) and the specificity is 95% (303/320). The low sensitivity of the Amsterdam Criteria II could be explained by the fact that the study population was largely unselected, in particular with respect to the family history. The high specificity is also likely to be attributable to the unselected nature of the population studied, with only a small proportion of families fulfilling the Amsterdam Criteria II. The positive predictive value of the Amsterdam Criteria II is only 32%. This may be explained by the fact that part of the mutation negative Amsterdam II families included only 3 colorectal cancer patients. Colorectal cancer occurs frequently in the general population and clustering of colorectal cancer by unknown genetic mechanism may occur.
Our data indicate that many mutations would be missed when relying entirely upon the Amsterdam Criteria II and that the decision to perform mutation analysis should not be exclusively dependent on fulfilling these criteria. In **Table 1** an approach is suggested to better select patients at risk for harboring an MMR germline mutation for mutation analysis. On the other hand, when a family fulfills the Amsterdam Criteria II, genetic analysis should be offered, of course.